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Characterization of the insulinotropic action of a phospholipase A_2 isolated from *Crotalus durissus collilineatus* rattlesnake venom on rat pancreatic islets

Tatiane C.A. Nogueira[a], Fabiano Ferreira[b], Marcos H. Toyama[a], Luiz Fabrizio Stoppiglia[a], Sergio Marangoni[a], Antonio C. Boschero[a,*], Everardo M. Carneiro[a]

[a]*Departamento de Fisiologia e Biofísica and Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, Campinas, SP 13083-970, Brazil*

[b]*Departamento de Fisiologia e Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Pernambuco (UFPE), Recife, PE, Brazil*

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Abstract

The ability of PLA_2 and crotapotin, isolated from *Crotalus durissus collilineatus* rattlesnake venom, to stimulate insulin secretion from isolated rat islets was examined. PLA_2 and crotapotin stimulated insulin secretion at 2.8 mmol/L glucose, whereas at a high glucose concentrations (16.7 mmol/L) only PLA_2 stimulated secretion. Nifedipine (10 μmol/L) did not alter the ability of PLA_2 to increase insulin secretion stimulated by a depolarizing concentration of K^+ (30 mmol/L). PLA_2 did not affect $^{14}CO_2$ production but significantly increased the efflux of arachidonic acid from isolated islets. These results indicate that PLA_2-stimulated secretion is not dependent on an additional influx of Ca^{2+} through L-type Ca^{2+}-channels but rather is associated with arachidonic acid formation in pancreatic islets.

Keywords: Arachidonic acid; Insulin secretion; Pancreatic islets; PLA2; Snake venom

1. Introduction

Several subspecies of *Crotalus durissus* occur in Brazil and account for 7–8% of venomous snakes in this country. *Crotalus durissus collilineatus* occurs in scrublands of central Brazil (States of Goiás, Minas Gerais and Tocantins) (Barraviera et al., 1989). Bites by *C. d. collilineatus* are characterized by neurotoxicity, systemic myotoxicity, edema, platelet aggregation, and acute renal failure, which is the most important cause of death (Ribeiro et al., 1998).

Crotoxin, the main neurotoxic component of *C. d. terrificus* venoms, consists of two subunits, crotapotin and PLA_2, with crotapotin acting as a chaperone molecule that enhances the neurotoxicity of the PLA_2 subunit at the neuromuscular junction (Bon, 1982). Crotoxin-like proteins have been found in other snake venoms, including *Crotalus scutulatus scutulatus* and *Crotalus viridis concolor* (Aird and Kaiser, 1985).

Phospholipases A_2 are a family of enzymes that catalyze the hydrolysis of glycerophospholipids at the sn-2 position to release free fatty acids, including arachidonic acid. PLA_2 plays a central role in several cellular processes, including metabolism, host defense and cellular signaling (Dennis, 1994). Multiple forms of PLA_2 have been found

* Corresponding author. Tel.: +55 19 3788 6202; fax: +55 19 3289 3124.

E-mail address: boschero@unicamp.br (A.C. Boschero).

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and classified in several groups according to their origin (secreted or cytosolic), primary structure, and disulfide bridge formation. The secreted PLA_2 from rattlesnake venom is a Ca^{2+}-dependent enzyme that belongs to group IIA (Six and Dennis, 2000).

Cytosolic Ca^{2+}-dependent and independent PLA_2s also occur in pancreatic B-cells (Jolly et al., 1993) and their ability to increase insulin secretion is related to the formation of arachidonic acid (Konrad et al., 1992a; Jones and Persaud, 1993). The inhibition of cytosolic PLA_2 decreases glucose-induced insulin secretion in islets (Konrad et al., 1992b), and the addition of cytosolic PLA_2 to the incubation medium increases insulin secretion from isolated rat islets (Zawalich and Zawalich, 1985).

In this work, we examined the effects of crotoxin and its subunits, crotapotin and PLA_2, on insulin secretion by isolated rat islets. At stimulatory concentrations of glucose, the ability of crotoxin to increase insulin secretion was related to PLA_2 activity and resulted in increased concentrations of arachidonic acid within the islet-cells.

2. Material and methods

Male Wistar rats (21 days old) from the breeding colony at the State University of Campinas (UNICAMP) were housed at 24 °C on a 12 h light/dark cycle, with access to a standard pellet diet and water ad libitum.

C. d. collilineatus venom was kindly donated by the Instituto Butantan (São Paulo, Brazil). All solvents and chemical reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Arachidonic acid, and ^{125}I human insulin were from Amersham (Aylesbury, UK). Sepharose protein A6 MB was from Pharmacia (Uppsala, Sweden).

2.1. Isolation of PLA_2

C. d. collilineatus venom was fractionated by molecular exclusion chromatography to obtain crotoxin that was purified further by reverse phase (RP) HPLC, as described by Toyama et al. (2001). Briefly, 25 mg of venom was dissolved in 0.2 M ammonium bicarbonate buffer, pH 7.8, and the solution was clarified by centrifugation at 4300*g* for 5 min. The supernatant was then applied to an HPLC column of Superdex 75 (1×60 cm) previously equilibrated with the same bicarbonate buffer. The elution profile was monitored at 280 nm and the fractions were tested for PLA_2 activity. The crotoxin obtained in this purification step was lyophilized and purified further by RP-HPLC. Three milligrams of crotoxin was fractionated by analytical RP-HPLC on a μ-Bondapack C18 column previously equilibrated with buffer A (0.1% trifluoroacetic acetic acid, TFA) for 15 min. The proteins were eluted using a linear gradient of buffer B (66.6% acetonitrile in 0.1% TFA). The resulting PLA_2 was assayed using bi-dimensional (2D) electrophoresis and MALDI-TOF mass spectrometry to confirm the purity of the material, as described elsewhere (Anderson et al., 1991).

2.2. Insulin secretion

Rat islets were isolated by collagenase digestion of the pancreas (Bordin et al., 1995). For static secretion, groups of five islets were first incubated for 45 min at 37 °C in Krebs-bicarbonate buffer of the following composition (in mmol/L): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 15 HEPES, and 5.6 glucose, supplemented with 3 g of bovine serum albumin/L and equilibrated with a mixture of 95% O_2–5% CO_2, pH 7.4. The medium was then replaced by fresh Krebs-bicarbonate buffer and the islets incubated for a further 1 h with medium containing different concentrations of glucose in the absence or presence of crotoxin (5.6 μg/mL), crotapotin (5.6 μg/mL), PLA_2 (5.6 μg/mL) and nifedipine (10 μmol/L), as indicated in the Results section. The insulin content of the medium at the end of the incubation period was measured by radioimmunoassay (Scott et al., 1981).

2.3. Glucose oxidation

Groups of 15 islets were transferred to polypropylene tubes with Krebs-bicarbonate solution containing D(U-^{14}C)glucose (0.5 mCi/mmol) and non-radioactive glucose at a final concentration of 16.7 mmol/L. The tubes were placed in 20 mL glass vials, equilibrated with 95% O_2–5% CO_2, sealed with a rubber membrane and incubated with slow shaking in a water bath at 37 °C for 2 h. At the end of the incubation period, 100 μL of 0.1 mol/L HCl was added to the tubes to stop the metabolism. Hyamine hydroxide (250 μL) was added to the outer of the vials to trap the CO_2 released by the islets and the vials were incubated for a further 1 h. At the end of the incubation, the polypropylene tubes and rubber membranes were removed and the radioactivity of the vials was determined in a beta counter (Beckman LS6000AT).

2.4. Arachidonic acid efflux

The efflux of arachidonic acid was determined as previously described (Simonsson et al., 1998). Briefly, islets were incubated overnight at 37 °C in an atmosphere of 95% O_2–5% CO_2 (pH 7.4) in 10 mL of RPMI 1670 medium, supplemented with 10% bovine serum albumin, 2.05 mmol/L L-glutamine, 100 IU of penicillin/mL, 100 μg of streptomycin B/mL and 4 μCi of [^{3}H]Arachidonic acid (specific activity: 209 Ci/mmol). Following an overnight incubation, the islets were washed in Krebs-bicarbonate buffer supplemented with 3% bovine serum albumin and 5.6 mmol/L glucose. Thereafter, groups of 25 islets were transferred into separate chambers and incubated for 1 h in 0.7 ml of Krebs-bicarbonate buffer supplemented with 3%

bovine serum albumin (37 °C, 95% O_2–5% CO_2, pH 7.4) and different concentrations of glucose, in the absence or presence of 5.6 μg of PLA_2/mL (Toyama et al., 2000).

After incubation, 0.5 mL of the medium was taken from each chamber and the radioactivity was measured in a beta counter (Beckman LS6000AT). The radioactivity remaining in the islets was also measured. The radioactivity in the medium was expressed as a percentage of the total islet radioactivity content.

2.5. Statistical analysis

The results were expressed as the mean ± SEM. Student's unpaired *t* test was used to compare the glucose oxidation and arachidonic acid efflux. When comparing the changes in insulin secretion, the data were log-transformed to correct for heterogeneity in variance and then analyzed by two-way ANOVA, followed by the Tukey–Kramer test to detect significant differences between groups and among glucose and secretagogue concentrations, and to assess the interactions between these factors. The data were analyzed using a statistical software package (Statsoft, Tulsa, OK, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Purification of PLA_2

Crotoxin from *C. d. colllineatus* venom corresponded to 47% of the venom protein obtained by molecular exclusion HPLC (Fig. 1a). Further purification of this component by RP-HPLC yielded eight major fractions. PLA_2, designated as Cdcolli F6, and two crotapotin fractions, are indicated in Fig. 1b. PLA_2 and crotapotin proteins corresponded to 56 and 34% of the crotoxin component, respectively. PLA_2 activity was analyzed using a specific PLA_2 assay, and the purity of the enzyme was confirmed by RP-HPLC, Tricine SDS-PAGE and mass spectrometry (data not shown).

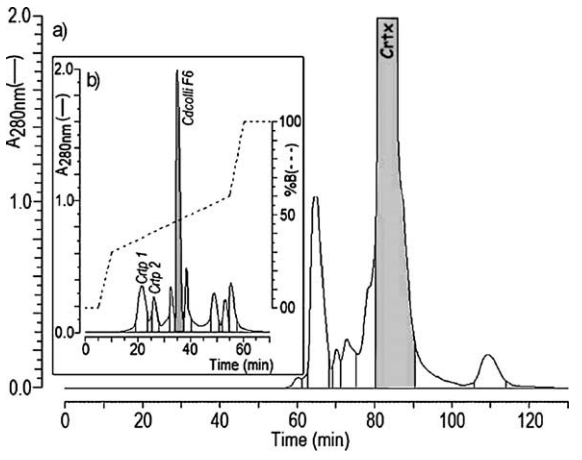

Fig. 1. (a) Molecular exclusion HPLC of *C. d. collilineatus* venom. The peak containing Crotoxin (Crtx) is indicated in gray and accounted for approximately 47% of the venom. (b) RP-HPLC of crotoxin on a α-Bondapack C18 column. The peaks corresponding to crotapotins 1 and 2 (crtp1, crtp2) and PLA_2 (Cdcolli F6) are indicated.

3.2. Effect of crotoxin, crotapotin and PLA_2 on insulin secretion and Arachidonic acid efflux from isolated islets

Crotoxin (5.6 μg/mL) significantly increased insulin secretion in the presence of 2.8 and 16.7 mmol/L glucose (Fig. 2A). Crotapotin (5.6 μg/mL) increased insulin secretion only in the presence of 2.8 mM glucose

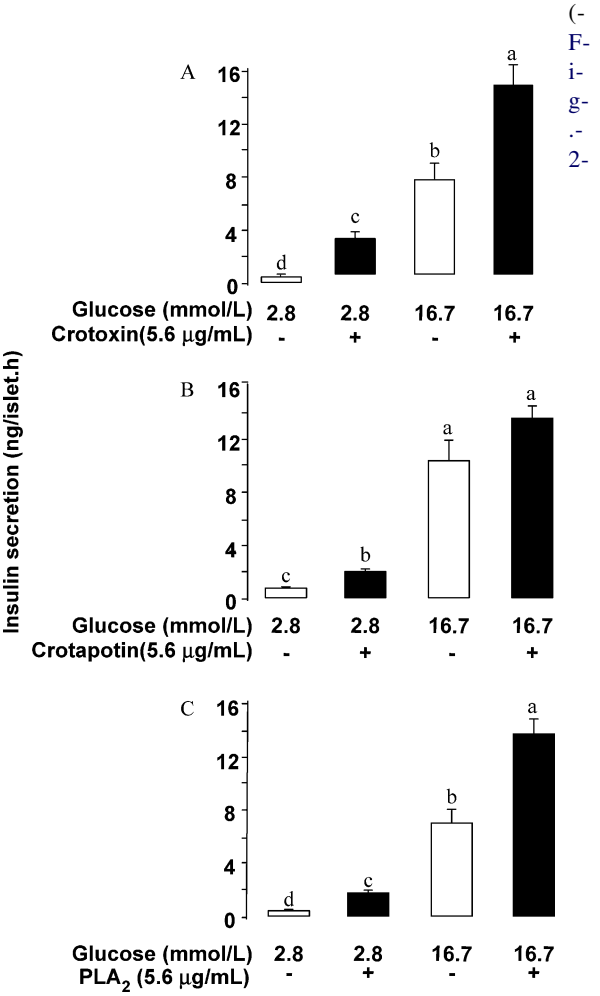

Fig. 2. Effect of crotoxin (A), crotapotin (B) and PLA_2 (C) on insulin secretion from isolated rat islets. Groups of five islets were incubated for 45 min in Krebs-bicarbonate medium containing 5.6 mmol/L glucose. The medium was subsequently replaced with Krebs solution containing 2.8 or 16.7 mmol/L glucose and 5.6 μg of crotoxin, crotapotin or PLA_2 per mL. The columns represent the cumulative insulin secretion during 1 h and are the mean ± SEM of 10 experiments. Means without a common letter differ. ($P < 0.05$).

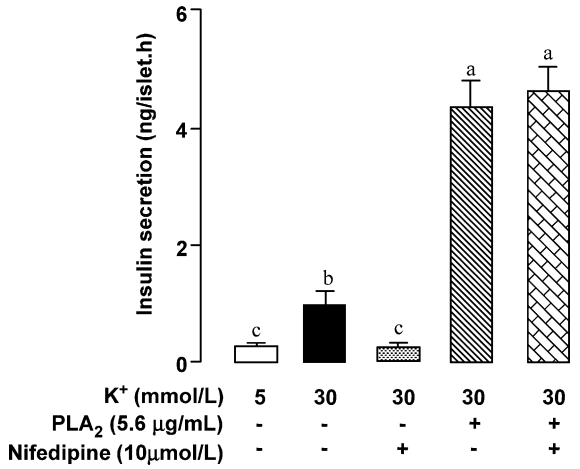

Fig. 3. Effect of nifedipine on insulin secretion by isolated islets. Groups of five islets were incubated for 45 min in Krebs-bicarbonate medium containing 5.6 mmol/L glucose. The medium was subsequently replaced with Krebs solution containing 30 mmol/L K^+ and nifedipine (10 µmol/L), as indicated. The columns represent the cumulative insulin secretion during 1 h and are the mean ± SEM of 10 experiments. Means without a common letter differ. ($P<0.05$).

B), whereas PLA_2 (5.6 µg/mL) induced a 4.2 and 1.9-fold increase in insulin secretion in the presence of 2.8 and 16.7 mmol/L glucose, respectively ($P<0.05$) (Fig. 2C). The insulin secretion induced by the association of PLA_2 (5.6 µg/mL) with high concentrations of K^+ (30 mmol/L) was unaffected by the Ca^{2+}-channel blocker nifedipine (Fig. 3).

The $^{14}CO_2$ production by islets was unaffected by PLA_2, averaging 32 ± 1.7 and 30 ± 0.6 pmol/islet 2 h in the presence and absence of PLA_2, respectively (data not shown).

The efflux of [^{3}H]Arachidonic acid from islets in the presence of PLA_2 was significantly higher than from control islets (Fig. 4A and B) ($P<0.05$). The increase in [^{3}H]Arachidonic acid efflux was accompanied by a concomitant increase in insulin secretion (Fig. 4C and D) ($P<0.05$).

4. Discussion

In this work, we examined the effect of crotoxin isolated from *C. d. collilineatus* venom, and its subunits crotapotin and PLA_2, on insulin secretion from isolated rat islets. Crotoxin significantly stimulated insulin secretion in the presence of low and high concentrations of glucose. At low concentrations of glucose (2.8 mmol/L), both subunits of crotoxin stimulated insulin secretion. However, in the presence of stimulatory concentrations of glucose (16.7 mmol/L) only PLA_2 stimulated release. Thus, the stimulatory capacity of crotoxin in the presence of high concentrations of glucose appears to be restricted to the PLA_2 subunit. Increase in the insulin secretion by the exposure to PLA_2 was already been described (Yamamoto et al., 1982; Zawalich and Zawalich, 1985). However, the mechanism of action of PLA_2 in inducing insulin secretion is still controversial. Recently, it was suggested that PLA2, released from B-cells by agents that increase cytosolic Ca^{2+} concentrations, strongly stimulated insulin secretion at basal or high concentrations of glucose via inhibition of the K^+_{ATP} channels (Juhl et al., 2003).

Glucose-induced insulin secretion depends on the metabolism of this sugar to increase the ATP/ADP ratio that in turn blocks K^+_{ATP} channels and causes B-cell membrane depolarization. The resulting massive influx of Ca^{2+}, mainly through the Ca^{2+} voltage-dependent L-type channels, increases the cytosolic Ca^{2+} concentration and stimulates insulin secretion (Petersen and Findlay, 1987; Grodsky, 1989).

Since the generation of ATP is essential for insulin secretion, we examined the effect of PLA_2 on glucose metabolism in isolated islets and found that PLA_2 did not alter the $^{14}CO_2$ production. Thus, stimulation of insulin secretion by PLA_2 was not caused by an alteration in glucose metabolism.

We also assessed the effect of nifedipine, a well-known L-type Ca^{2+} channel blocker, on insulin secretion stimulated by a high concentration of K^+. As shown in Fig. 3, PLA_2 increased insulin secretion in the presence of a depolarizing concentration of K^+ (30 mmol/L). Nifedipine blocked the insulin secretion induced by a high concentration of K^+ in the absence but not in the presence of PLA_2. These results indicate that PLA_2-stimulated secretion was not due to an additional increase in the Ca^{2+} influx into B-cells. Apparently, these results contrast with the former observation that D600, another Ca^{2+} channel blocker, significantly reduced the insulin secretion induced by PLA_2 (Zawalich and Zawalich, 1985). However, they are in accordance with more recent observation that PLA_2 does not modify depolarization-induced exocytosis (Juhl et al., 2003).

Since PLA_2 causes tissue damage (Curin-Serbec et al., 1991; Pungecar et al., 1999) PLA_2 may have increased the insulin secretion by damaging the B-cell membrane. To exclude this possibility, pancreatic islets were incubated with PLA_2 for 1 h and then exposed to 16.7 mmol/L glucose. The ability of the islets to secrete insulin following this treatment was similar to that found in control islets, thus ruling out the possibility that PLA_2 stimulated secretion by damaging B-cells (data not shown). In addition, the integrity of the plasma membrane of B-cells was not altered by the exposure of the islets to PLA_2, as judged by trypan blue exclusion technique (data not shown).

In another series of experiments, we examined the effect of PLA_2 on arachidonic acid efflux from perifused islets. PLA_2 significantly increased the arachidonic acid efflux from islets in the presence of low and high concentrations of

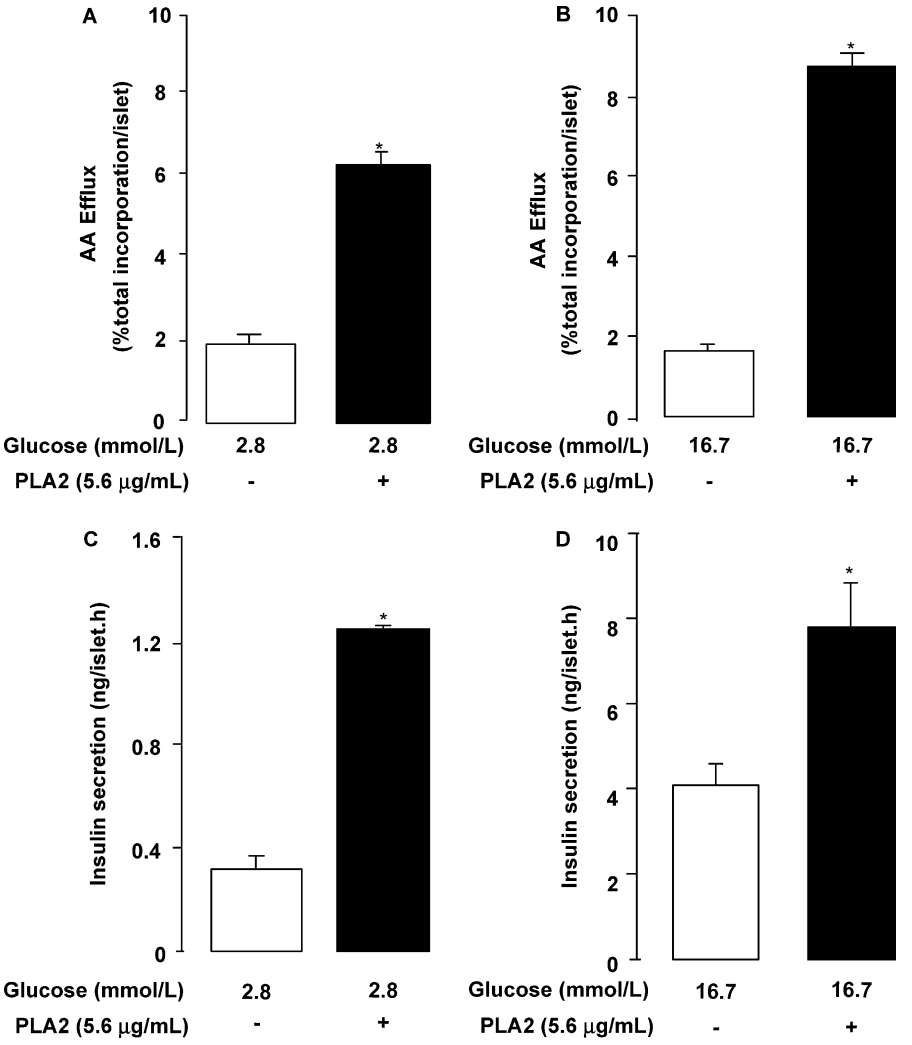

Fig. 4. Arachidonic acid efflux (A and B) and insulin secretion (C and D) by isolated islets. The islets were incubated overnight in RPMI medium containing [^{3}H]Arachidonic acid and then extensively washed with medium containing no radioisotope and further incubated for 1 h in Krebs-bicarbonate medium containing 2.8 or 16.7 mmol/L glucose in the absence or presence of PLA_2. The columns represent the arachidonic acid efflux and insulin secretion and are the mean ± SEM of 12 experiments. *Different from the control group. ($P < 0.05$).

glucose (Fig. 4A and B). In parallel, there was an increase in the insulin secretion (Fig. 4C and D). These data agree with reports showing that arachidonic acid may increase insulin secretion via an extracellular, Ca^{2+}-independent pathway (Band et al., 1992). A possible interaction between PLA_2 and the cell membrane leading to phospholipid breakdown and the formation of prostaglandin and arachidonic acid has been suggested (Smith and Waite, 1992; Suga et al., 1993). The ability of arachidonic acid to increase insulin secretion appears to be a consequence of its action at several sites including a possible interaction with K^+ channels (Ordway et al., 1989; Juhl et al., 2003), and the stimulation of adenylyl cyclase (Engelhard et al., 1978), guanylyl cyclase (Gerzer et al., 1985), PKC (Metz, 1988), and Ca^{2+}-calmodulin kinase (Piomelli and Greengard, 1991).

In conclusion, the insulinotropic capacity of crotoxin isolated from *C. d. collilineatus* venom is associated with its PLA_2 subunit. The increase in insulin secretion is probably related to arachidinic acid formation by PLA_2 within B-cells and is independent of an increase in cytosolic Ca^{2+} following the entry of extracellular Ca^{2+}.

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